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PRINCIPAL INVESTIGATOR: John A.A. Ladas, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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14. ABSTRACT The prostate apoptosis response-4 (Par-4) protein induces apoptosis in prostate cancer cells but not in normal cells. The region spanning residues 145–203 of human Par-4 kills androgen-dependent and androgen-independent cancer cells but not normal cells, and is termed selective for apoptosis induction in cancer cells (SAC) domain. Par-4 also contains a C-terminal coiled-coil (CC) domain that interacts with the protein kinase Akt1 and the DNA-binding domain of WT1. Phosphorylation of Par-4 by Akt1 results in inhibition of apoptosis. To obtain insights into the mechanisms of Par-4 selective killing of prostate cancer cells, we expressed the human Par-4 SAC domain in bacteria and purified it to homogeneity. Numerous attempts to crystallize this protein in the apo form failed to yield diffraction-quality crystals. Analysis of the SAC domain using NMR spectroscopy revealed that it is unstructured, demonstrating the need to perform a structural analysis of this domain bound to its binding partner, where SAC will likely adopt a structure. The structures of Par-4 SAC and CC domains will facilitate the elucidation of the mechanisms underlying the selective killing of prostate cancer cells by this protein and will guide the development of novel molecular targeted therapies for prostate cancer.					
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INTRODUCTION

Prostate cancer starts as a localized androgen-dependent disease and progresses to a metastatic and often androgen-independent disease. This progression is due to the lack of an apoptotic response to androgen-ablation despite the presence of intact apoptotic programs within the androgen-independent cancer cells. The prostate apoptosis response-4 (Par-4) protein can override the anti-apoptotic mechanisms and induce apoptosis in prostate cancer cells but not in normal cells (1). Remarkably, the region spanning residues 145–203 of human Par-4 induces apoptosis to both androgen-dependent and androgen-independent cancer cells but not normal cells, and is termed selective for apoptosis induction in cancer cells (SAC) domain (2). Phosphorylation of human Par-4 at T163 by protein kinase A is required for activation of the SAC apoptotic potential in cancer cells (3). Par-4 also contains a C-terminal leucine zipper or coiled-coil (CC) domain (Figure 1A) that interacts with the protein kinase Akt1 (4) and the zinc finger DNA-binding domain of WT1 and regulates the Bcl-2 gene expression by WT1 in prostate cancer cells (5). Importantly, phosphorylation of Par-4 by Akt1 results in cytoplasmic retention of Par-4 by the protein 14–3–3 and inhibition of apoptosis (4). These findings demonstrate that the Par-4 SAC and CC domains are prime targets for developing novel molecular targeted therapies against prostate cancer. This project focuses on the elucidation of the three-dimensional structures of the Par-4 SAC and CC domains with the goal to obtain insights into the mechanisms of Par-4 selective killing of prostate cancer cells.

BODY

During the 18-month award period, we proceeded with the experiments proposed in the Statement of Work. A description of our progress in these studies follows below.

Task 1. To express, purify, and crystallize the Par-4 and WT1 domains. In order to perform a structural analysis of the Par-4 SAC and CC domains in the apo form and in complex with their protein targets, it is necessary to produce large amounts (multi-milligram quantities) of these domains in bacterial cells as soluble proteins and purified them to homogeneity. Toward this goal, the DNA fragments encoding the human Par-4 SAC domain (amino acid residues 141–203) and the region spanning residues 248–340 that encompasses the CC domain were amplified by the polymerase chain reaction (PCR) method, using the human Par-4 cDNA (clone BC000591, ATCC MGC-815) as a template. The resulting DNA fragments were cloned using standard protocols (6) into prokaryotic expression vectors pET31b and pET-6H2, a modified pET-16b vector (Novagen) that allows the expression of recombinant hexahistidine-tagged protein in *Escherichia coli* cells, as we described previously (7). A site for the TEV protease (ENLYFQ↓G) was engineered between the hexahistidine-tag and the N-terminal end of the Par-4 domains to allow proteolytic removal of the tag following purification of the recombinant proteins. The resulting constructs were verified by DNA sequencing and were used to transform *E. coli* BL1(DE3) cells. Following induction with IPTG, the expressed proteins were tested for solubility. The SAC domain was purified to homogeneity using a combination of affinity (on Ni-NTA resin), ion exchange, and size exclusion chromatography (Figure 1B). However, the Par-4 CC domain was expressed in the insoluble fraction (inclusion bodies). Numerous attempts to

produce this domain as a soluble protein in many *E. coli* strains in a wide range of temperatures and in different culture media were unsuccessful. Therefore, we spent a major effort during the entire period of the award for the production of soluble Par-4 CC domain and not so much effort in the expression of the zinc finger DNA-binding domain of WT1 because in the absence of the Par-4 CC domain the WT1 protein is of no use for the completion of the goals of this project. Following the purification of the Par-4 SAC domain, we initiated crystallization experiments of this protein in the apo form using the vapor-diffusion sitting-drop method. Despite an extensive effort to crystallize this domain, we obtained only microcrystals (Figure 1D) that cannot be used for crystallographic analysis.

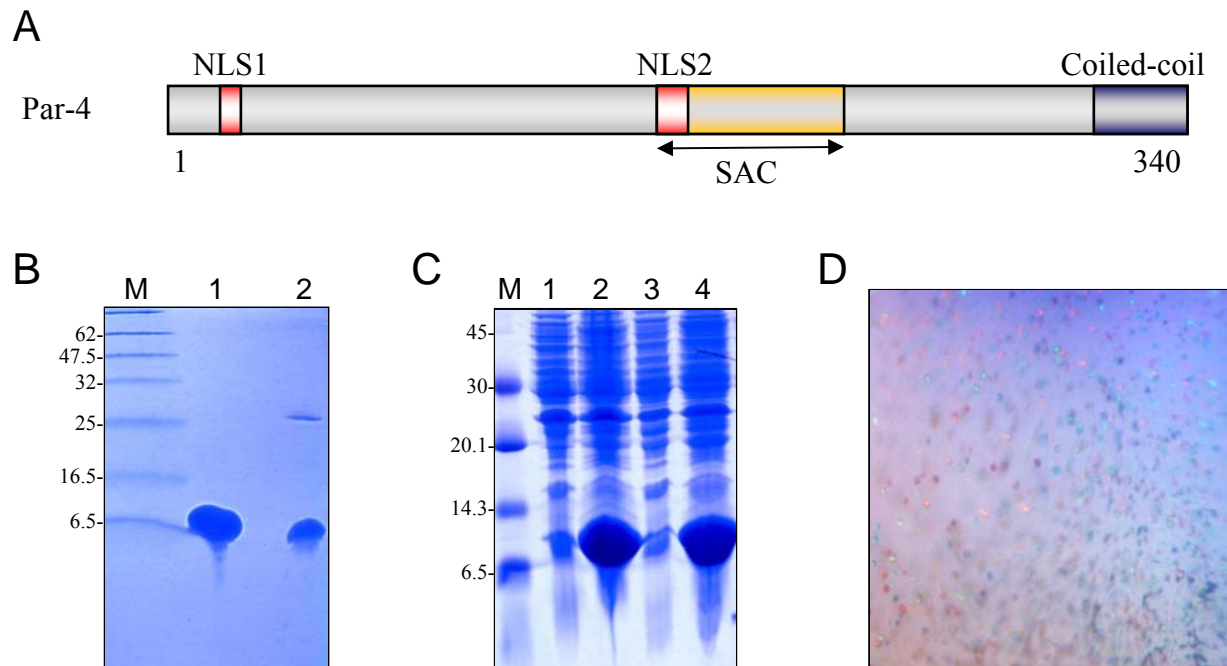


Figure 1. (A) Schematic representation of Par-4 SAC and CC domains that were expressed in bacterial cells. (B) Expression of the Par-4 SAC domain in *E. coli* BL21(DE3) cells. Lane M: protein markers in kDa; lanes 1 and 2: fractions of eluted Par-4 SAC protein following purification with size exclusion chromatography. (C) Expression of the Par-4 CC domain in *E. coli* BL21(DE3) cells. Lane M: protein markers in kDa; lanes 1 and 2: uninduced and induced whole-cell extracts, respectively, of *E. coli* cells expressing Par-4 CC protein; lane 3: induced soluble proteins; lane 4: induced insoluble proteins. (D) Microcrystals of the Par-4 SAC protein using the sitting-drop method.

Task 2. To determine the crystal structures of the Par-4 and WT1 domains. Because the Par-4 SAC domain failed to yield diffraction-quality crystals, we used nuclear magnetic resonance (NMR) spectroscopy to obtain structural information on the SAC domain, an approach often taken for proteins that are refractory to crystallization. We produced ^{15}N -labeled SAC protein in *E. coli* cells grown in M9 minimal media using ^{15}N -labeled ammonium sulfate as the sole nitrogen source, and purified it to homogeneity using affinity, ion exchange, and size exclusion chromatography. Heteronuclear single quantum correlation (HSQC) spectra of the

SAC protein were recorded on a Bruker 600 MHz instrument equipped with a cryoprobe (Bruker BioSpin Corporation, Billerica, MA), as we described previously (8). The protein sample is stable at a relatively high concentration (~0.25 mM) under NMR conditions and displays a spectrum with a narrow range of backbone amide protons (~1 ppm) (Figure 2). These studies indicate that the Par-4 SAC domain is unstructured in the unbound form and demonstrate the need to perform a structural analysis of this domain in complex with its putative binding partner(s), where SAC will likely adopt a structure. Experiments are underway to identify proteins that interact with the SAC domain and also purify the Par-4 CC domain and crystallize it in complex with the WT1, Akt1, and 14-3-3 proteins.

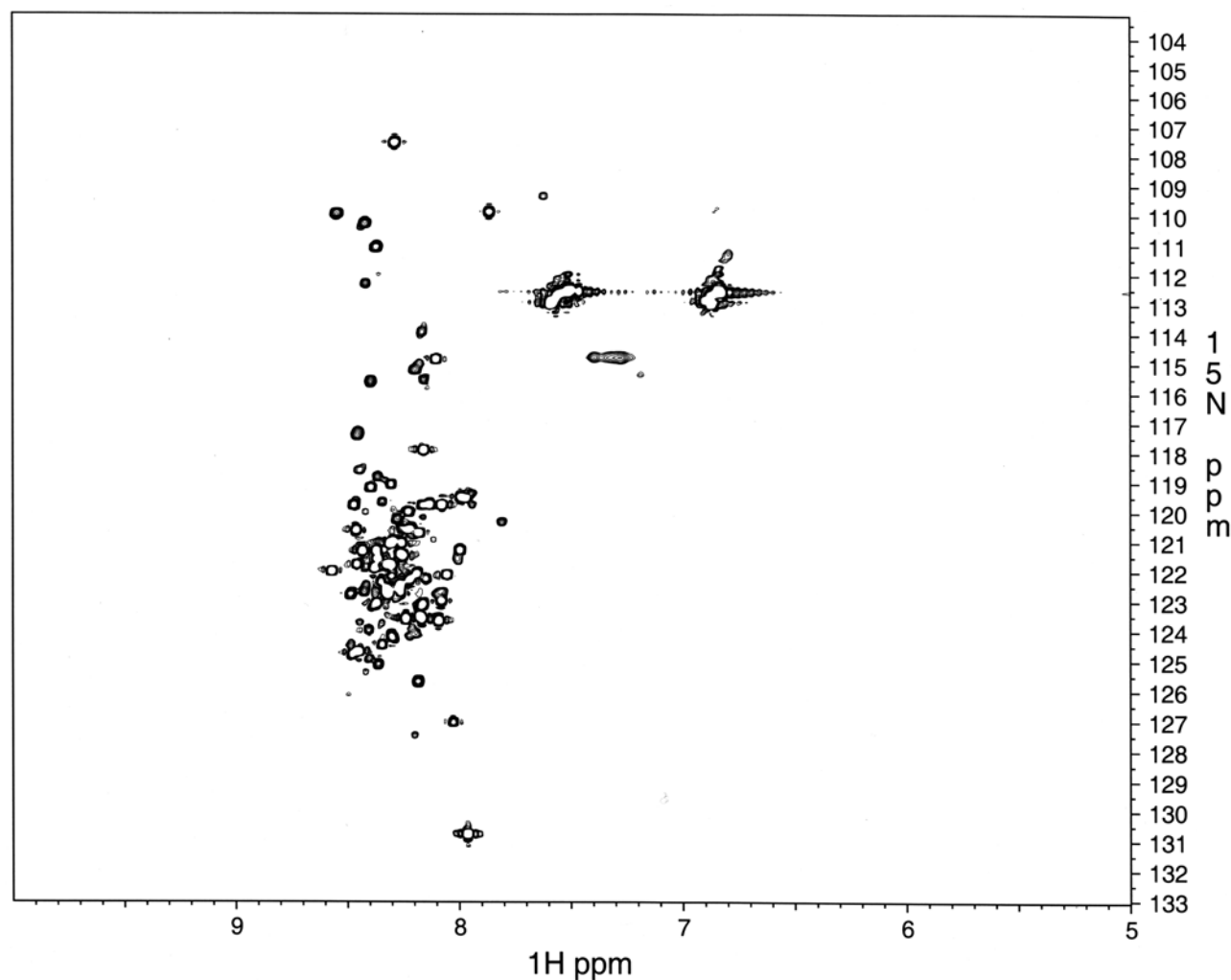


Figure 2. Contour plot of the HSQC spectrum of the purified Par-4 SAC domain acquired at 298 K. The spectrum was recorded on a Bruker 600 MHz instrument equipped with a cryoprobe. For 256 transients in the indirect dimension 16 scans were acquired with 1,024 points each. The resulting data set was zero-filled once in each dimension before Fourier transformation and final processing. The sample was in a buffer consisting of 50 mM potassium phosphate, pH 6.5, supplemented with 100 mM NaCl and trace sodium azide.

KEY RESEARCH ACCOMPLISHMENTS

1. Cloning of the human Par-4 SAC domain (residues 141–203) and the Par-4 CC domain (residues 248–340) into prokaryotic expression vectors pET31b and pET-6H2 for production in *E. coli* cells (*Task 1*).
2. Induction of expression and performance of solubility tests of the bacterially produced Par-4 SAC and CC proteins (*Task 1*).
3. Large-scale production of Par-4 SAC protein in *E. coli* cells for crystallization and NMR studies (*Task 1*).
4. Crystallization experiments of the Par-4 SAC purified protein (*Task 1*).
5. Large-scale production of ¹⁵N-labeled Par-4 SAC protein in *E. coli* cells for solution structure studies using NMR (*Tasks 1 and 2*).
6. Recorded NMR HSQC spectra of the ¹⁵N-labeled Par-4 SAC protein for solution structure studies using NMR (*Tasks 1 and 2*).

REPORTABLE OUTCOMES

The experiments of the proposal are still in progress and no publications have resulted so far.

CONCLUSIONS

During the award period we cloned, expressed in bacteria, and purified the human Par-4 SAC domain to homogeneity. We made numerous attempts to crystallize this protein in the apo form but we were not able to obtain diffraction-quality crystals for structural analysis. In an attempt to circumvent this problem, we produced and purified ¹⁵N-labeled Par-4 SAC protein for structural analysis in solution using NMR spectroscopy. HSQC spectra of the SAC protein showed that it is unstructured in the unbound form, demonstrating the need to perform a structural analysis of this domain in complex with its putative binding partners, where SAC will likely adopt a structure. In parallel, we spent a long time trying to optimize the conditions for the production of large amounts of the Par-4 CC domain in a soluble form suitable for structural studies. Although the award period is finished, we will continue to pursue very persistently the structural analysis of the Par-4 SAC and CC domains, because the atomic structures of these domains will be extremely helpful in the elucidation of the molecular mechanisms underlying the remarkable ability of this protein to selectively kill prostate cancer cells. The SAC structure will provide a framework for the rational design of small molecules that would mimic the function of this domain and/or enhance its apoptotic activity in cancer cells, whereas the CC structure would guide the development of drugs that would inhibit the interaction of Par-4 with Akt1 and/or 14–3–3 proteins and induce selecting apoptosis in prostate cancer cells. This award has provided critical support to initiate this research project and the successful completion of these studies will be reported to DOD in the future.

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